Plant Physiol. (1997) 113: 535–548

Distinction between Endoplasmic Reticulum-Type and Plasma Membrane-Type Ca^{2+} Pumps[1]

Partial Purification of a 120-Kilodalton Ca^{2+}-ATPase from Endomembranes

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Two biochemical types of Ca^{2+}-pumping ATPases were distinguished in membranes that were isolated from carrot (*Daucus carota*) suspension-cultured cells. One type hydrolyzed GTP nearly as well as ATP, was stimulated by calmodulin, and was resistant to cyclopiazonic acid. This plasma membrane (PM)-type pump was associated with PMs and endomembranes, including vacuolar membranes and the endoplasmic reticulum (ER). Another pump ("ER-type") that was associated mainly with the ER hydrolyzed ATP preferentially, was insensitive to calmodulin, and was inhibited partially by cyclopiazonic acid, a blocker of the animal sarcoplasmic/ER Ca^{2+} pump. Oxalate stimulation of Ca^{2+} accumulation by ER-type, but not PM-type, pump(s) indicated a separation of the two types on distinct compartments. An endomembrane 120-kD Ca^{2+} pump was partially purified by calmodulin-affinity chromatography. The purified polypeptide bound calmodulin reacted with antibodies to a calmodulin-stimulated Ca^{2+} pump from cauliflower and displayed [^{32}P]phosphoenzyme properties that are characteristic of PM-type Ca^{2+} pumps. The purified ATPase corresponded to a phosphoenzyme and a 120-kD calmodulin-binding protein on endomembranes. Another PM-type pump was suggested by a 127-kD PM-associated protein that bound calmodulin. Thus, both ER- and PM-type Ca^{2+} pumps coexist in most plant tissues, and each type can be distinguished from another by a set of traits, even in partially purified membranes.

Transient increases in cytosolic [Ca^{2+}] is an important intracellular signal for many stimuli-induced responses. The diverse array of stimuli include elicitors, light, temperature, and hormones (Bush, 1995). Cytosolic [Ca^{2+}] increases when these stimuli cause the transient opening of one or more Ca^{2+} channels on the PMs or endomembranes. Millimolar levels of Ca^{2+} (0.1–1 mM) in the extracellular space and in endomembrane compartments flow down their electrochemical gradient into the cytosol, which usually maintains a [Ca^{2+}] of 0.2 to 0.6 μM. The resulting increase in [Ca^{2+}] to as much as 1 to 10 μM usually terminates within a few minutes (Bush, 1995). Although direct evidence for the events leading to a [Ca^{2+}] decrease in the cytosol has not been demonstrated in plants, Ca^{2+} pump activity can control the frequency of Ca^{2+} waves induced by a chemical signal in frog oocytes (Camacho and Lechleiter, 1993). Hence, in plants Ca^{2+}-pumping ATPases and H^+-coupled Ca^{2+} transporters can be activated in response to an increase in cytosolic [Ca^{2+}]. These energy-dependent Ca^{2+} transporters extrude cytosolic Ca^{2+} to the external medium or into internal stores, such as the vacuole and the ER, and thus maintain low cytosolic [Ca^{2+}]. Because the affinity for Ca^{2+} of Ca^{2+}-ATPases is higher than that of the antiporter (Bush and Sze, 1986; Bush, 1995), the Ca^{2+} pumps are thought to play the major role in fine-tuning intracellular [Ca^{2+}].

In addition to a role in intracellular signaling, cellular Ca^{2+} is essential for the functioning of the secretory system. In mammalian cells many membrane proteins and soluble proteins destined for secretion are synthesized, folded, and assembled at the ER. The correct folding and assembly depends on ER chaperones such as calnexins, which require Ca^{2+} for activity (Bergeron et al., 1994). Ca^{2+} chelators abolish the ability of calnexin to associate with proteins, and Ca^{2+} ionophores disrupt the regulation of the secretory system (Sambrook, 1990). These results suggest that changes in the intraluminal [Ca^{2+}] can disturb the mechanisms for protein folding, assembly, and secretion. This model is supported by studies using yeast *pmr1* mutants that are deficient in a Ca^{2+} pump on the Golgi. Significantly, *pmr1* mutants secrete proteins that are retained in the ER in wild-type cells (Rudolph et al., 1989). In plants secretory materials (e.g. extracellular proteins) are synthesized, folded, and assembled in the ER, passed through the Golgi, and then transferred to the PM or the vacuole. Along these routes each step is vesicle-mediated and involves vesicle recognition, docking, and fusion. Similarly, vacuole expansion during cell elongation is depen-

[1] This work was supported in part by a grant from the Department of Energy to H.S. and in part by the Maryland Agricultural Experiment Station.

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Abbreviations: BiP, a major ER-resident binding protein; BTP, bis-Tris-propane or 1,3-bis(Tris[hydroxymethyl]methylamino)-propane; CaM, calmodulin; PE, phosphoenzyme; PM, plasma membrane; PMCa, animal plasma membrane-type Ca^{2+}-pumping ATPase; SER, sarcoplasmic/ER; SERCa, animal SER-type Ca^{2+}-pumping ATPase; TPCK, *N*-tosyl-L-Phe chloromethyl ketone; VM23, a tonoplast intrinsic protein of approximately 23 to 25 kD.

dent on continuous vesicle trafficking and fusion. It is well documented that Ca^{2+} is an important component of vesicle trafficking and fusion in plants (Battey and Blackbourn, 1993); thus, intraluminal Ca^{2+} as well as cytosolic Ca^{2+} could participate in the normal operation of the secretory system in plants as in animals. Sufficient levels of luminal Ca^{2+} would depend on the activity and regulation of both Ca^{2+} pumps and H^+/Ca^{2+} antiporters that are localized on endomembranes.

Considering the activities of the secretory system during plant cell division and cell expansion, one working model predicts that Ca^{2+} pumps could reside on the PM, ER, vacuolar membrane, Golgi, and perhaps other (secretory) vesicles. Some Ca^{2+} pumps could be related, and some may be distinct in their structure and their mode of regulation. The current literature illustrates the diversity of Ca^{2+} pumps in plants; however, there are apparent inconsistencies and puzzling questions regarding the biochemical nature, the membrane location, and the relationship among the various pumps (Evans et al., 1991). The uncertainties are caused in part by the different plant materials used, especially since some tissues/organs are enriched for one particular Ca^{2+} pump. For example, a prominent CaM-stimulated Ca^{2+} pump on the PM of radish seedlings hydrolyses ATP, GTP, or ITP (Rasi-Caldogno et al., 1992). However, it is not clear whether this pump is related to another CaM-stimulated Ca^{2+} pump that is localized to the tonoplast in barley or corn roots (DuPont et al., 1990; Gavin et al., 1993). The animal ER-type Ca^{2+} pump is distinguished from the animal PM-type Ca^{2+} pump by its sensitivity to thapsigargin or cyclopiazonic acid, and by its insensitivity to CaM (Schatzmann, 1989; Siedler et al., 1989; Carafoli 1992); however, the results from plants are more ambiguous. For example, Ca^{2+} pump activity in the ER fraction is insensitive to CaM in wheat aleurone layers (Bush and Wang, 1995) and in garden cress (Buckhout, 1984), yet other studies show high CaM-stimulated Ca^{2+} transport in endomembranes, including the ER from carrot (*Daucus carota*) suspension cells (Hsieh et al., 1991), cauliflower florets (Askerlund and Evans, 1992), corn roots (Brauer et al., 1990), and tendrils (Liss and Weiler, 1994).

To distinguish among the multiple pumps in one experimental system, we have used various biochemical tools to separate and identify Ca^{2+} pumps in carrot suspension-cultured cells. These cells are actively proliferating during the exponential growth phase and are highly secretory. Low-density membrane vesicles isolated from carrot suspension-cultured cells were abundant in vanadate-sensitive Ca^{2+} pump activity (Bush and Sze, 1986). The transport activity was stimulated 2- to 3-fold by CaM and co-migrated with an ER marker on Suc gradients (Hsieh et al., 1991). A Ca^{2+} pump of 120 kD was identified by the formation of a Ca^{2+}-dependent PE. Because PE formation was stimulated by La^{3+}, we concluded that a 120-kD phosphoprotein represented a PM-type pump, even though it was localized on endomembranes such as the ER (Chen et al., 1993). The presence of other Ca^{2+} pumps in carrot membranes was considered (Hsieh et al., 1991), although our results were insufficient to clearly distinguish one Ca^{2+} pump from another. Here we show how two major types of Ca^{2+} pumps can be distinguished by their biochemical characteristics independently of the purity of the membrane. One type of pump was energized by GTP or ATP and stimulated by CaM. This type of activity was located on endomembranes (such as the vacuole and ER) as well as the PM. An endomembrane Ca^{2+}-ATPase of 120 kD was purified by CaM-affinity chromatography and represented a member of this type. Another type of Ca^{2+} pump hydrolyzed ATP preferentially but was insensitive to CaM. This type of activity was located mainly on the ER. The results are consistent with the working model in which distinct types of Ca^{2+} pumps are required for supplying luminal Ca^{2+} into diverse endomembrane compartments and for regulating cytosolic $[Ca^{2+}]$ in response to a host of signals received by proliferating cells.

Although the concept of two general classes of Ca^{2+} pumps has been discussed in plants for several years (Briskin, 1990; Evans et al., 1991), an ER-associated pump or a PM-associated pump was usually studied independently using separate plant materials. In a few cases, two Ca^{2+} pump types were examined in either purified ER or PM fractions from a single plant material (Briskin, 1990; Thomson et al., 1993; Bush and Wang, 1995). Important traits emerged (e.g. substrate preference and erythrosin B sensitivity) from each study, although diagnostic tools to distinguish one pump activity from a membrane mixture were lacking. Here we have taken another approach. Instead of separating pumps by their membrane association, we have differentiated Ca^{2+} pumps by their biochemical characteristics. The advantage of such an approach is that features of one pump type are revealed independently of its location. Consequently, (a) a set of traits identified by us and others can be used as a diagnostic tool for distinguishing between two major types of Ca^{2+} pumps, and (b) activity from either ER- or PM-type pumps can be determined in membranes containing a mixture of pump types. To our knowledge, the complete characterization and the ability to distinguish between two major types of Ca^{2+} pumps, independently of their location, has not been carried out previously in a single species.

MATERIALS AND METHODS

Carrot (*Daucus carota* var Danvers) cells were grown in suspension in Murashige-Skoog medium (Murashige and Skoog, 1962) supplemented with 1 mg/L 2,4-D and 0.1 mg/L 6-BA. Cells were subcultured weekly, and membranes were isolated from 5- to 6-d-old cells that were in the exponential phase of growth.

Isolation of Membrane Vesicles

Membranes were isolated from carrot suspension-cultured cells as described by Chen et al. (1993) with some modification. Briefly, carrot cells were homogenized in 250 mM Suc, 25 mM Hepes-BTP (pH 7.4), 3 mM EGTA, 1 mM PMSF, 0.1 mM TPCK, 1 mM DTT, and 0.5% BSA. The homogenate was centrifuged at 1,000*g* for 10 min and then at 7,500*g* for 20 min. The supernatant (7 mL) was layered on

a discontinuous Suc gradient and centrifuged at 100,000*g* for 2 h. The gradient consisted of 5 mL each of 15 and 22% Suc over a 6-mL cushion of 32% Suc in 25 mM Hepes-BTP (pH 7.4), 1 mM DTT, and 1 mM PMSF. Endomembranes at the 22/32% Suc interface were suspended in a medium containing 25 mM Hepes-BTP (pH 7.4), 10% glycerol, 100 mM KCl, 1 mM DTT, and 1 mM PMSF and stored at −80°C. The stored membrane vesicles were used for activity assays or for further solubilization and purification.

Sometimes 7 mL of a post-mitochondrial supernatant from 5 g of cells was separated with a linear 10 to 40% Suc gradient (24 mL) over a 45% Suc cushion (3 mL). After centrifugation at 100,000*g* for 3 h, fractions (1.3–1.5 mL each) were used for various activity assays. For phosphoprotein determinations, an aliquot (0.5 mL) of each fraction was diluted to 5 mL with 10% Suc in 25 mM Hepes-BTP (pH 7.0), 1 mM DTT, and 1 mM PMSF and then pelleted and suspended in 0.2 mL of the same buffer solution.

Protein concentration was estimated using the Bio-Rad protein assay or the Bradford method after incubation of samples with 0.1% Triton. BSA was used as the standard.

Solubilization and Purification

Vesicles at the 22/32% Suc interface were diluted 20-fold with 25 mM Hepes-BTP (pH 7.0), 100 mM KCl, 3 mM EGTA, 1 mM DTT, 0.1 mM TPCK, and 1 mM PMSF and incubated on ice for 40 min. The washed vesicles were concentrated by pelleting through a 10-mL 15% (w/w) Suc cushion at 100,000*g* for 1 h at 4°C. The Suc cushion enhanced the removal of residual BSA. Pellets were then suspended to approximately 2.5 mg/mL in suspension medium. Usually 5 to 7 mg of protein (approximately 2 mL) was recovered from an initial 90 g fresh weight of cells.

Prior to the addition of detergent, fresh DTT and protease inhibitors were added to the EGTA-washed vesicles to final concentrations of 3 mM DTT, 1 mM PMSF, 0.1 mM TPCK, 0.1 mM pepstatin a, and 1 mM chymostatin. Then, a 5-fold stock solution (0.5 mL) of Triton/lipid/$CaCl_2$ was added dropwise to the resuspended vesicles (2 mL) with gentle vortexing. The final mixture for solubilization contained 2 mg/mL protein, 1% Triton X-100, 5 mM $CaCl_2$, 10% glycerol, 25 mM Hepes-BTP (pH 7), 100 mM KCl, and 0.1 mg/mL asolectin. The mixture was incubated for 40 min (4°C) with continuous, gentle mixing and then centrifuged at 156,000*g* (60,000 rpm, Beckman TL100.3 rotor, 1-mL tubes) for 20 min. The supernatant, containing solubilized protein, was supplemented with 25 μL of 50 mg/mL asolectin, 15 μL of 0.5 M DTT, and 1 μL of 0.5 M PMSF (final concentrations of 0.5 mg/mL, 3 mM, and 0.2 mM, respectively), and used immediately for CaM-affinity chromatography.

CaM-affinity chromatography was performed as originally described by Niggli et al. (1979) with some modifications. Solubilized protein (3–6 mg of protein) from EGTA-washed vesicles was allowed to bind to a 2-mL CaM-Sepharose-affinity column (Pharmacia, Piscataway, NJ). The column was equilibrated prior to loading with a 5 mM Ca^{2+} column buffer (10% glycerol, 25 mM Hepes-BTP [pH 7.0], 100 mM KCl, 5 mM $CaCl_2$, 0.04% Triton, 0.5 mg/mL asolectin, 1 mM DTT, and 0.1 mM PMSF). Solubilized protein was loaded at 20 mL/h (4°C) and material passing through the column was reloaded directly onto the column for 40 min, equal to at least four reloadings of the unbound material. The column was initially washed with 20 to 40 mL of column buffer containing 5 mM $CaCl_2$ and 2-mL fractions were collected. The column was then washed with 6 to 8 mL each of column buffers containing decreasing $CaCl_2$ concentrations of 2, 1, 0.5, 0.25, and 0.1 mM. Bound protein was then eluted with a column buffer containing 2 mM EGTA. Immediately after collection, 10 mM $CaCl_2$ was added to EGTA-eluted fractions. In some experiments Ca^{2+} was added to one-half of each EGTA-eluted fraction. Fractions lacking Ca^{2+} were used as controls to estimate background counts per minute in PE assays. The EGTA-eluted and the washed fractions were sometimes concentrated 5- to 10-fold by ultrafiltration with a Centricon-30 unit (Amicon, Beverly, MA).

PE Activity and Acidic SDS-PAGE

To assay for the steady-state levels of PE (Chen et al., 1993), aliquots (50 μL) of membrane vesicles, solubilized protein, or column fractions were incubated with [γ-^{32}P]ATP or GTP (Amersham) at 4°C for 2 min. Components of the final reaction mixtures (typically 200 μL) were equal to that of the 5 mM Ca^{2+} column buffer (above) plus 2 nM [^{32}P]ATP and 100 μM $LaCl_3$. The reaction was stopped with an equal volume of a 2-fold stop solution (100 mM NaH_2PO_4, 2 mM ATP, and 20% TCA). After 30 min at 4°C, the tubes were centrifuged to collect TCA-precipitated ^{32}P-phosphoproteins. The pellets were washed by vortexing with 1 mL of a 1-fold stop solution and re-pelleted. Pellets were resuspended in 2% SDS and transferred to vials for scintillation counting. Because PE formation was completely inhibited by 5 μM erythrosin (Chen et al., 1993), 5 μM erythrosin (Sigma, E-7379) was used to determine the background counts per minute for Ca^{2+}-containing samples. Sometimes the background counts per minute were determined by adding 5 or 20 mM EGTA to EGTA-eluted fractions or to fractions that did not bind to the column, respectively. Thus, "PE activity" as used here refers to either erythrosin-sensitive PE or Ca^{2+}-dependent PE.

For SDS-PAGE analysis of ^{32}P-phosphoproteins, the TCA-pelleted protein was suspended in a sample buffer (2.5 mM sodium phosphate [pH 6.3], 2.5% [w/v] lithium dodecyl sulfate, 0.5% [v/v] β-mercaptoethanol, 0.25 mg/mL bromphenol blue, and 25% glycerol). Samples were separated on a 5% acrylamide minigel buffered with phosphate (100 mM NaH_2PO_4/Na_2HPO_4 [pH 6.3], 0.1% SDS) at 50 V for 4 h at 24°C (Weber and Osborn, 1969). Gels were dried onto filter paper and placed in cassettes with XAR-5 film (Kodak) for autoradiography.

SDS-PAGE and Silver Staining of Proteins

Protein was solubilized in an equal volume of a 2-fold sample buffer containing 125 mM Tris-HCl (pH 6.8), 2% SDS, 20% (v/v) glycerol, 8 M urea, 10% 2-mercaptoethanol, and 0.004% (w/v) bromphenol blue. The proteins were

separated on a 7.5 or 12% acrylamide gel at pH 8.8 (15 × 20 cm) and at 9 mA per gel overnight at 15°C. Gels were either silver-stained for proteins or electroblotted.

Immunostain

After electrophoresis, proteins were blotted onto Immobilon-P (Millipore) in 25 mM Tris (pH 8.3), 192 mM Gly, and 20% methanol at 50 V for 4 h at 4°C. The Immobilon-P was blocked in TPBS (PBS with 0.1% Tween 20) containing 5% dry milk and 1% protease-free BSA (Sigma) for 1 h and washed three times with TPBS. The membrane was incubated for 1 h with polyclonal antibodies diluted with TPBS containing 1% BSA and then washed. The membrane was probed with goat anti-rabbit IgG (Calbiochem) conjugated to alkaline phosphatase, and color was developed with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium. Antibodies to BiP, an ER lumen protein, the PM H^+-ATPase, the vacuolar membrane VM23, and the cauliflower Ca^{2+}-ATPase were generously provided by M. Chrispeels (University of California, San Diego), R.T. Leonard (University of Arizona, Tucson), M. Maeshima (Hokkaido University, Sapporo, Japan), and P. Askerlund (Lund University, Sweden), respectively.

Binding to Biotinylated CaM

Biotinylated CaM was used to detect membrane or solubilized proteins that bound CaM directly (Kincaid et al., 1988). After SDS-PAGE, proteins were blotted onto Immobilon-P in 25 mM Tris (pH 8.3), 192 mM Gly, and 20% methanol. The Immobilon-P was blocked with 1% BSA in 50 mM Tris (pH 7.5), 200 mM NaCl, 0.5 mM $CaCl_2$, and 50 mM $MgCl_2$ (Tris-buffered saline/CaMg). The blot was incubated with 100 ng/mL biotinylated CaM (Calbiochem) in the same buffer for 2 h at 22°C and washed twice with Tris-buffered saline/CaMg containing 0.05% Tween 20. Binding was detected after incubation with streptavidin conjugated to alkaline phosphatase, and color development was with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (Sigma). To detect nonspecific CaM binding, electroblotted proteins were incubated with biotinylated CaM in the presence of 2 mM EGTA to remove Ca^{2+}. Although detection of ^{125}I-CaM binding to proteins (Ling and Assmann, 1992) was more sensitive than of biotinylated CaM, this method was discontinued because of potential hazards.

Ca^{2+} Transport

Ca^{2+} uptake at 22°C was measured with $^{45}CaCl_2$ by the filtration method (Hsieh et al., 1991). Transport was usually initiated by adding 20 to 40 μL of vesicles (20–40 μg of protein) to a reaction mixture (final volume 0.25 mL) containing 200 mM Suc, 25 mM Hepes-BTP (pH 7.0), 10 mM KCl, 0.1 mM NaN_3, 5 mM KNO_3, 10 μM $^{45}CaCl_2$ (0.5 μCi/mL), 3 mM $MgSO_4$, and 3 mM ATP (or 3 mM GTP) with or without 2 μM CaM (bovine brain, Sigma P-2277). Aliquots (0.2 mL) from duplicate reactions were filtered and washed with 2 mL of cold rinse solution (250 mM Suc, 2.5 mM Hepes-BTP [pH 7.0], and 0.2 mM $CaCl_2$). The $^{45}Ca^{2+}$ that was retained on the filters was determined by liquid scintillation counting. The net active transport at 15 min was determined as the difference in activity in the presence and absence of Mg^{2+}.

To examine the effect of cyclopiazonic acid (Sigma), membranes were preincubated with the inhibitor for 10 min at 22°C. Ca^{2+} transport was initiated by adding 10 μM $^{45}CaCl_2$ (0.5 μCi/mL) and 0.6 mM ATP or GTP. Background Ca^{2+} associated with vesicles was estimated in a reaction mixture with 10 μM erythrosin B in the absence of other inhibitors.

Chemicals

The lipid used in all solutions was asolectin (45% phosphatidylcholine) from Avanti Polar Lipids (Alabaster, AL). The lipid was suspended in deionized H_2O and sonicated under N_2 before addition to aqueous solutions. All other chemicals were of reagent grade.

RESULTS

ATP-Dependent and GTP-Driven Ca^{2+} Transport Represents Two Separate Ca^{2+} Transport Activities

To distinguish multiple Ca^{2+} pumps in carrot cells, we determined the Ca^{2+} pump activity in membrane fractions using either GTP or ATP as the substrate. Previous studies had shown that the PM-bound (Rasi-Caldogno et al., 1995), but not the ER-associated Ca^{2+} pump from plants hydrolyzed GTP in addition to ATP (Briskin, 1990). Most of the GTP-driven Ca^{2+} pump activity was associated with the vacuolar membranes (17–22% Suc). In contrast, the maximum ATP-driven Ca^{2+} uptake was found in the ER vesicles (24–27% Suc) (Fig. 1A). The differential distribution of the GTP-driven and ATP-driven activities was not altered by CaM (Fig. 1B). The vacuolar membrane, PM, and ER were clearly separated, as verified by immunostaining with antibodies to marker proteins (Fig. 2B). VM23, a vacuolar membrane integral protein (Maeshima, 1992), and the PM-H^+-ATPase peaked at 15 to 22% Suc and at 36 to 41% Suc, respectively. The distribution of BiP, an ER lumen chaperone, although broad, peaked at 24 to 29% Suc, similar to that of NADH Cyt *c* reductase (25–28% Suc) as shown previously (Hsieh et al., 1991).

The differential pattern of Ca^{2+} transport driven by GTP and by ATP indicated the presence of multiple Ca^{2+} pumps with a differential substrate preference. We consistently noted that ATP-driven transport was either equal to (Fig. 1, A and B, fractions 1 and 2 and 14–18) or higher than GTP-driven Ca^{2+} transport (fractions 3–13). One simple interpretation is that one pump hydrolyzed GTP nearly as well as ATP, and another pump preferred ATP over GTP. Although other interpretations are possible, this simple model is supported by the following results.

ATP-Preferred Ca^{2+} Transport Is Inhibited by Cyclopiazonic Acid and Is Insensitive to CaM

Assuming one pump hydrolyzed GTP nearly as well as ATP, then GTP-driven Ca^{2+} transport would represent one

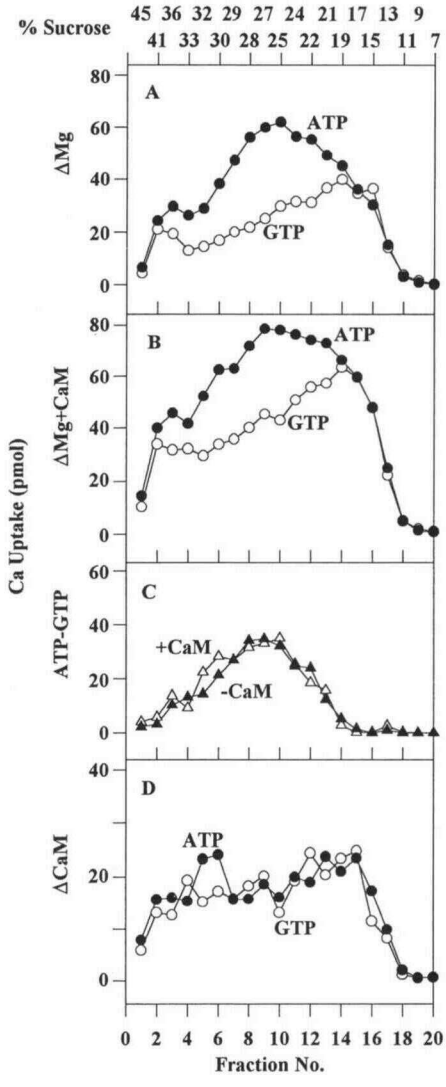

Figure 1. Differential distribution of GTP-driven and ATP-driven Ca^{2+}-pumping activities in membranes from carrot cells. The post-mitochondrial supernatant was separated with a linear 10 to 40% Suc gradient and fractionated. ATP-driven (●) and GTP-driven (○) $^{45}Ca^{2+}$ transport was measured in the absence (A) or presence (B) of 2 μM CaM. ATP-preferred Ca^{2+} uptake (C) was estimated by subtracting GTP-driven from the ATP-dependent Ca^{2+} transport in either the absence (▲) or presence (△) of CaM. CaM-stimulated transport (D) driven by either GTP (○) or ATP (●) was calculated from the difference in activity with or without CaM. One experiment is representative of three.

biochemical type of the Ca^{2+} pump. Activity from another type of Ca^{2+} pump that preferred ATP over GTP could then be estimated from the difference in activity driven by ATP and by GTP. The pump that preferred ATP was possibly associated with the ER because its activity peaked at 25 to 28% Suc (Figs. 1C and 2B; Hsieh et al., 1991). It is interesting that the distribution and activity of the pump that preferred ATP was unaltered by CaM (Fig. 1C), indicating that this type of Ca^{2+} pump was not regulated directly by CaM.

Another feature of ATP- but not GTP-driven Ca^{2+} transport was the enhancement by oxalate (Fig. 3, A and B). Oxalate stimulation of Ca^{2+} uptake is thought to be caused by formation of Ca^{2+}-oxalate precipitate inside the vesicles, thus decreasing the magnitude of the Ca^{2+} chemical gradient (Bush and Sze, 1986). If so, the stimulation of Ca^{2+} uptake by oxalate would depend on an oxalate carrier that co-localized to the same compartment membrane as the Ca^{2+} pump. Most of the oxalate-stimulated Ca^{2+} transport was found in the ER at 24 to 30% Suc, with a minor component possibly in the Golgi (31% Suc) (Fig. 3, A and C). In contrast, GTP-driven Ca^{2+} transport in a range of membrane compartments was unaffected by oxalate (Fig. 3, B and C), perhaps because oxalate carriers were absent from these membranes.

If Ca^{2+} accumulation that was enhanced by oxalate and CaM-stimulated Ca^{2+} transport were located on separate membrane compartments, then the net Ca^{2+} uptake that was enhanced by oxalate would be insensitive to CaM. However, if an oxalate carrier and a CaM-stimulated Ca^{2+} pump resided on the same compartment, then CaM-stimulated activity would also be elevated by oxalate. We found that CaM had no effect on the Ca^{2+} accumulation that was enhanced by oxalate and that oxalate had no effect

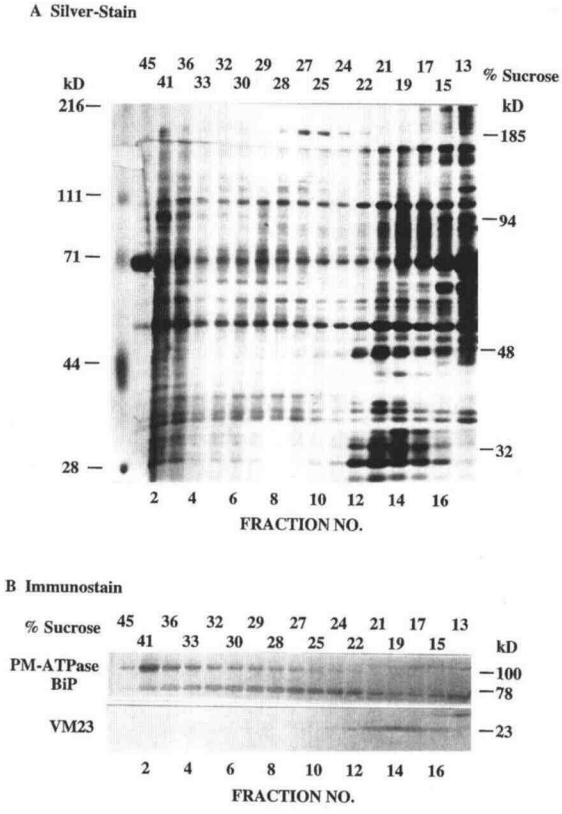

Figure 2. The vacuolar membrane and the PM markers peaked at 15 to 22% Suc and 36 to 41% Suc, respectively. The post-mitochondrial supernatant was separated with a linear Suc gradient as in Figure 1. Equal-volume fractions (4–8 μg of protein) were separated by SDS-PAGE (7.5 or 12% acrylamide). A, Silver-stained gel. B, Immunostaining with antibodies to vacuolar membrane VM23, to PM H^+-ATPase, and to BiP, an ER lumen protein.

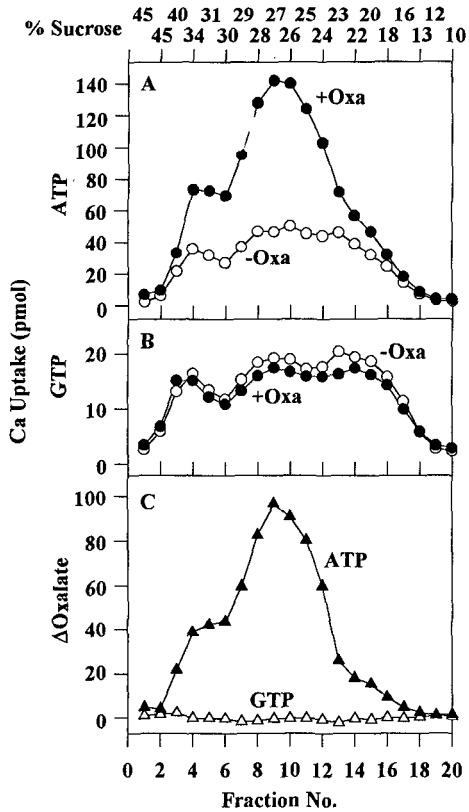

Figure 3. Stimulation by oxalate of ATP-driven (A), but not of GTP-driven (B), Ca^{2+} transport. The post-mitochondrial supernatant was separated on a linear Suc gradient as described in Figure 1. Active Ca^{2+} uptake was measured with (+Oxa, ●) or without 10 mM potassium oxalate (−Oxa, ○) in the presence of 10 mM KNO_3. Oxalate-stimulated ATP-driven Ca^{2+} uptake (C) was determined by subtracting activity without oxalate from that with oxalate. One experiment is representative of two.

on CaM-stimulated Ca^{2+} transport driven by ATP (Table I). Thus, Ca^{2+} accumulation enhanced by oxalate was driven by a CaM-insensitive, ATP-preferred pump. Furthermore, the ATP-preferred Ca^{2+} pump and the CaM-stimulated Ca^{2+} transport (see next section) were located on separate compartments.

More importantly, oxalate-stimulated ATP-dependent Ca^{2+} transport activity was inhibited by cyclopiazonic acid at concentrations (100 nmol/mg protein; Fig. 4) that specifically block animal SERCa-type pumps (Siedler et al., 1989). Because ATP protects the enzyme in a competitive manner (Siedler et al., 1989), the cyclopiazonic acid effect on Ca^{2+} transport was tested with 0.6 mM substrate instead of 3 mM. Under these conditions, cyclopiazonic acid consistently inhibited ATP-driven Ca^{2+} transport activity by 20 to 34% but not GTP-driven transport (Fig. 4). Together these results demonstrate that ER-associated Ca^{2+} transport could be inhibited by cyclopiazonic acid and was insensitive to CaM. As a working model, we refer to this pump as the "ER-type" to distinguish it from GTP-driven Ca^{2+} transport.

Table I. *Separation of oxalate-stimulated Ca^{2+} accumulation and CaM-stimulated Ca^{2+} uptake in endomembrane vesicles from carrot cells*

Net Mg^{2+}-dependent Ca^{2+} uptake (15 min) was determined as described in Fig. 1. Reaction mixtures contained vesicles from the 22/32% Suc interface, 20 mM KNO_3 with or without 10 mM K_2-oxalate. CaM stimulated transport (ΔCaM) was calculated from the difference in activity with (+CaM) and without (−CaM) CaM.

Substrate	Net Ca^{2+} Uptake		
	−CaM	+CaM	ΔCaM
	nmol mg^{-1} protein (%)		
ATP			
−Oxalate	4.53 (100)	6.42 (142)	1.89 (42)
+Oxalate	11.95 (100)	13.74 (115)	1.79 (15)
ΔOxalate	7.42 (100)	7.32 (99)	0 (0)
GTP			
−Oxalate	1.77 (100)	3.09 (175)	1.32 (75)
+Oxalate	1.27 (100)	2.66 (209)	1.39 (109)

GTP-Driven Ca^{2+} Transport Is CaM-Stimulated

Regardless of the substrate that was used, GTP or ATP, the distribution and level of CaM-stimulated Ca^{2+} transport was unchanged (Fig. 1D). Thus, CaM was activating the same type of pump(s), and GTP-dependent transport alone could represent this type of activity. The net Ca^{2+} pumped by GTP or by ATP was strikingly similar in low-density membranes (13–19% Suc) and in the membranes of 41% Suc (Fig. 1B), suggesting an enrichment of this pump type in vacuolar membranes and PMs. Although the vacuolar H^+-ATPase also utilizes GTP as a substrate, activity from the vacuolar H^+/Ca^{2+} antiport was relatively low in the endomembrane vesicles from carrots (Bush and Sze, 1986). Furthermore, bafilomycin had little or no effect on Ca^{2+} pumping (data not shown). Thus, we will refer to

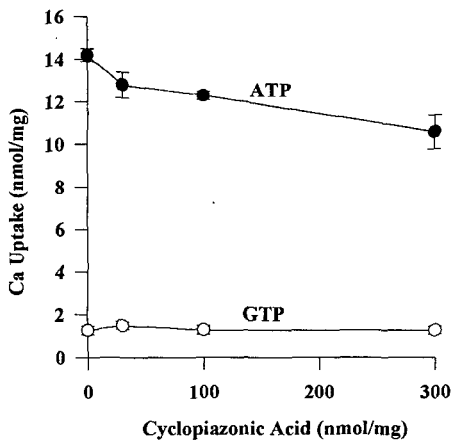

Figure 4. Differential effect of cyclopiazonic acid on ER- and PM-type Ca pumps in endomembranes. Net Ca^{2+} uptake (15 min) was determined in reaction mixtures that contained vesicles from the 22/32% Suc interface, 20 mM KNO_3, 0.6 mM ATP (or 0.6 mM GTP) with or without 10 mM potassium oxalate. Oxalate-stimulated Ca^{2+} uptake was plotted for ATP-dependent transport only. Results are an average of three experiments. Error bars indicate ±SE. With 7 to 10 μg protein 250 μL^{-1} reaction mixture, a concentration of 300 nmol cyclopiazonic acid mg^{-1} protein is equivalent to 12 μM.

GTP-driven Ca^{2+} transport as activity from "PM-type" Ca^{2+} pump(s). Since the vacuolar membrane and PM were well separated (Fig. 2B), the broad distribution of CaM-stimulated Ca^{2+} transport indicated that this type of pump was associated with several membranes of the secretory system. The gradient in Figure 1 was intentionally overloaded to permit several assays from the same gradient. When fewer membranes were loaded per gradient, CaM-stimulated or GTP-driven transport was consistently resolved into several peaks, including low-density vacuolar membranes (20–23% Suc), PMs (36–38% Suc), and endomembranes such as the ER (26–27% Suc; Fig. 3).

PE of 120 kD Formed with ATP or GTP Is Localized on Various Membranes

With [γ-^{32}P]ATP, a major 120-kD PE was formed in membranes ranging in density from 20% Suc through 42% Suc (Fig. 5A), consistent in general with the distribution of ATP-driven Ca^{2+} transport (Figs. 1 and 3). Because ATP-dependent PE formation could reflect activities from both the ER- and PM-type Ca^{2+} pumps, the fraction corresponding to each type could not be resolved. A GTP-dependent [^{32}P]PE of 120 kD was also formed in membranes ranging in density from 22 to 41% Suc (Fig. 5B). It is interesting that the level of PE was not strictly coincident with GTP-driven or CaM-stimulated Ca^{2+} transport (Figs. 1 and 3). The steady-state PE level was highest in membranes at 27 to 28% Suc and very low in light-density membranes (15–20% Suc; Fig. 5B). It is possible that the assay condition that was used was favorable for detecting PE formation from one of several Ca^{2+} pumps. It is also important to note that the steady-state level of a PE is not a direct measure of the reaction rate (Schatzmann, 1989), thus, PE levels might not be quantitatively related to transport activity. The results demonstrate that phosphorylated-type Ca^{2+}-pumping ATPase(s) of 120 kD were localized on various membranes, including the PM and several endomembranes.

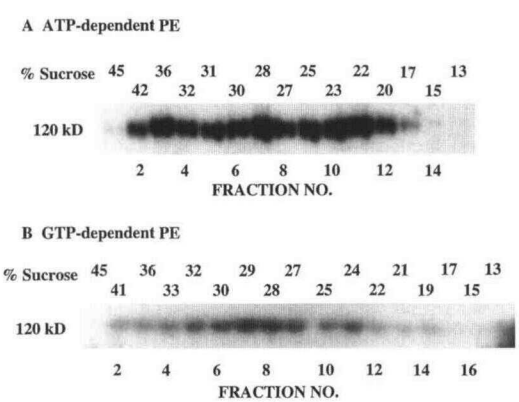

Figure 5. A PE of 120 kD was localized on several endomembranes and the PM in parallel with Ca^{2+} transport. The post-mitochondrial supernatant was separated with a linear Suc gradient as in Figure 1. Membrane fractions (4–8 μg of protein) were incubated with either 2 nM [γ-^{32}P]ATP (A) or 2 nM [γ-^{32}P]GTP (B) for 2 min at 4°C in the presence of 50 μM Ca^{2+} and 100 μM $LaCl_3$. The TCA-precipitated ^{32}P-phosphoproteins were separated on a 6% acrylamide gel at pH 6.3 and visualized using autoradiography. One experiment is representative of three.

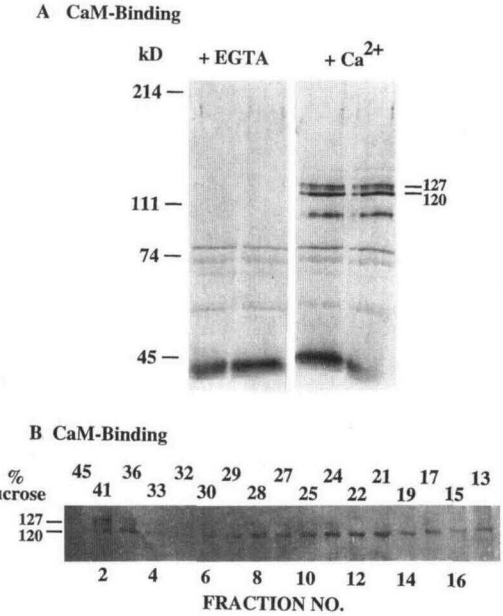

Figure 6. A 120-kD polypeptide associated with endomembranes bound to biotinylated CaM. A, Ca^{2+}-dependent CaM-binding proteins of carrot membranes. Ten micrograms of protein from the 22/32% Suc interface was separated on a 7.5% acrylamide gel and electroblotted. Electroblotted proteins were incubated with biotinylated-CaM in the presence of 2 mM EGTA (left) or 0.5 mM $CaCl_2$ (right). Bound CaM was visualized with streptavidin conjugated with alkaline phosphatase. B, Distribution of the 120-kD CaM-binding protein in membranes separated on a linear Suc gradient. CaM binding was determined with 50 μL (4–8 μg of protein) of each membrane fraction.

CaM-Binding Polypeptides of 120 and 127 kD Are Associated with Endomembranes and PMs

Membrane proteins were tested for their ability to bind biotinylated CaM. Several proteins of 110 to 127 kD from low-density membranes (22/32% Suc) bound to CaM in a Ca^{2+}-dependent manner (Fig. 6A). After membranes were separated with a Suc gradient, a major CaM-binding protein of 120 kD was detected in the vacuolar membrane fraction (21–22% Suc), in endomembranes (24–25% Suc), and in the PM fraction (36–41% Suc; Fig. 6B). Additional CaM-binding polypeptides of 125 to 127 kD were associated with the PM. The similar distribution of a 120-kD CaM-binding protein, GTP-driven Ca^{2+} transport activity (Fig. 3B), and a 120-kD PE (Fig. 5) supports the idea that a 120-kD CaM-binding protein could be a Ca^{2+}-ATPase.

Solubilization of PE Activity

To further identify and characterize a CaM-binding Ca^{2+} pump from endomembranes, a Ca^{2+} pump was purified by CaM-affinity chromatography (Niggli et al., 1979) using membranes collected from the 22/32% Suc interface. Triton effectively solubilized PE activity from membrane vesicles

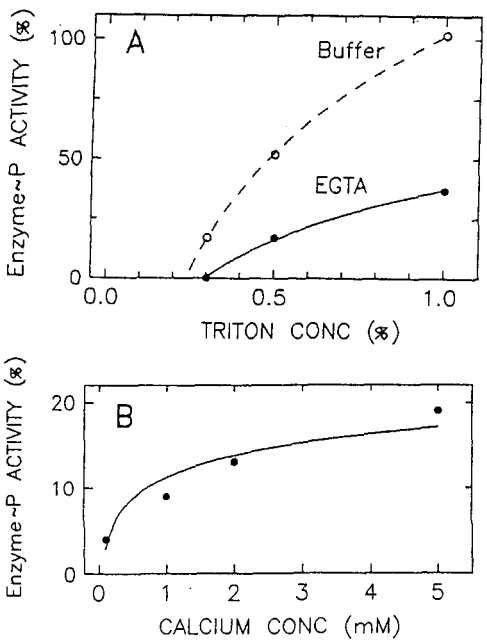

Figure 7. Solubilization of PE activity by Triton from buffer-washed (○) and EGTA-washed (●) membrane vesicles. A, Effect of detergent concentration. Membrane vesicles were incubated with buffer or buffer containing 3 mM EGTA and pelleted prior to solubilization. For solubilization, washed vesicles (2 mg/mL) were incubated in a solubilization mixture containing 0.3, 0.5, or 1% Triton. After the sample was centrifuged, PE activity in the supernatant was determined and expressed as a percentage of the PE activity of washed vesicles. The results shown are from one representative experiment with each treatment in duplicate. B, Effect of added Ca^{2+} during solubilization. EGTA-washed vesicles were solubilized as described above except that the solubilization solution contained 0.5 mg/mL asolectin, 1% Triton, and [$CaCl_2$] as indicated.

(Fig. 7A). Because solubilized protein would be applied to a CaM-affinity column, we washed native membrane vesicles with EGTA to remove endogenous CaM prior to solubilization. When vesicles were washed with 3 mM EGTA prior to solubilization, however, recovered PE activities were always well below that obtained with buffer-washed vesicles (Fig. 7A); typically, 5 to 25% of the PE activity in membrane vesicles was recovered in the solubilized fraction. The poor recovery of activity suggested that the enzyme(s) were inactivated by EGTA or degraded by proteolysis or both. The addition of millimolar Ca^{2+} during solubilization was required (Fig. 7B). PE activity, once solubilized, was stable on ice for periods up to 48 h when 0.5 mg/mL asolectin and 5 mM Ca^{2+} were present (data not shown).

Partial Purification of a CaM-Binding Ca^{2+}-ATPase of 120 kD

To determine whether a Ca^{2+}-ATPase that was detected as a PE directly bound to CaM, solubilized protein from EGTA-washed vesicles was applied to a 2-mL CaM-Sepharose column equilibrated with 5 mM Ca^{2+}. Proteins that contain CaM-binding domains will bind to a CaM-affinity column in the presence of Ca^{2+} and are eluted upon removal of Ca^{2+} from the column solution (Carafoli, 1991). At least 80% of the PE activity consistently remained unbound (Fig. 8A; Table II), despite numerous attempts to increase binding by adjusting binding conditions (see "Discussion"). After the column was washed to remove the unbound protein and to reduce Ca^{2+} in the column solution to 0.1 mM, bound protein was eluted with 2 mM EGTA. A peak of PE activity representing 0.3 to 2% of the starting activity eluted from the column in coincidence with the EGTA treatment (Fig. 8A). The partially purified fraction had an estimated 30- to 40-fold enrichment of PE-specific activity in comparison with solubilized PE activity (Table II). The major proteins that bound to the CaM-affinity column were of 120, 68, 54, 50, and 44 kD (Fig. 8B, lanes 8–10); however, only a 120-kD polypeptide was phosphorylated by [γ-^{32}P]ATP in the presence of Ca^{2+} in the EGTA-eluted fraction (Fig. 9A, lanes 5 and 6). The results indicate that a 120-kD Ca^{2+}-ATPase was partially purified by CaM-affinity chromatography.

EGTA eluted a CaM-binding protein of 120 kD (Fig. 9B, lanes 5–7) in parallel with a phosphoprotein of similar molecular mass (Fig. 9A). The fractions that did not bind to the CaM-affinity column also showed some CaM-binding activity (Fig. 9B, lanes 1–3). Using densitometry, we estimated that approximately 15% of the CaM-binding activity at 120 kD was recovered in the EGTA-eluted fractions. The identity of a 120-kD polypeptide as a Ca^{2+} pump was

Table II. *Partial purification of a carrot Ca^{2+}-ATPase using CaM-affinity chromatography*

The unbound and bound/EGTA eluate correspond to fractions 1 to 6 and 26 to 32, respectively, in Figure 8A. PE activity was measured as Ca^{2+}-dependent PE at 2 min in the presence of 2 nM [^{32}P]ATP and 100 μM $LaCl_3$. Protein values were determined by the Bradford method except for the bound/EGTA eluate for which protein was estimated from silver-stained SDS-PAGE gels. Results are from one experiment representative of six. The percentage of recovery was calculated relative to either starting membranes or total solubilized protein (in parentheses).

Purification Step	Total Protein	[^{32}P]PE Activity			
		Total activity	Recovery	Specific activity	-Fold
	mg	*fmol*	*%*	*fmol mg^{-1}*	
Membranes (22/32% Suc)	4.5	403	100	89	1
1% Triton supernatant	0.8	72	18 (100)	90	
CaM-Sepharose					
Unbound	0.8	64	16 (89)	80	
Bound/EGTA Eluate	0.001	2.46	0.6 (3.4)	2457	28

further confirmed by its reactivity with polyclonal antibodies (Fig. 9C, lanes 5–7) to an endomembrane Ca^{2+} pump that was purified from cauliflower (Askerlund, 1996). Collectively, these results demonstrate that we have partially purified a CaM-binding Ca^{2+}-ATPase of 120 kD.

Properties of the Partially Purified Ca^{2+}-ATPase

The nature of the PE activity that bound to the CaM-affinity column and was eluted with EGTA appeared similar to the PE activity in carrot endomembrane vesicles as characterized previously (Chen et al., 1993). Total PE activity was Ca^{2+}-dependent (Table III) and inhibited by vana-

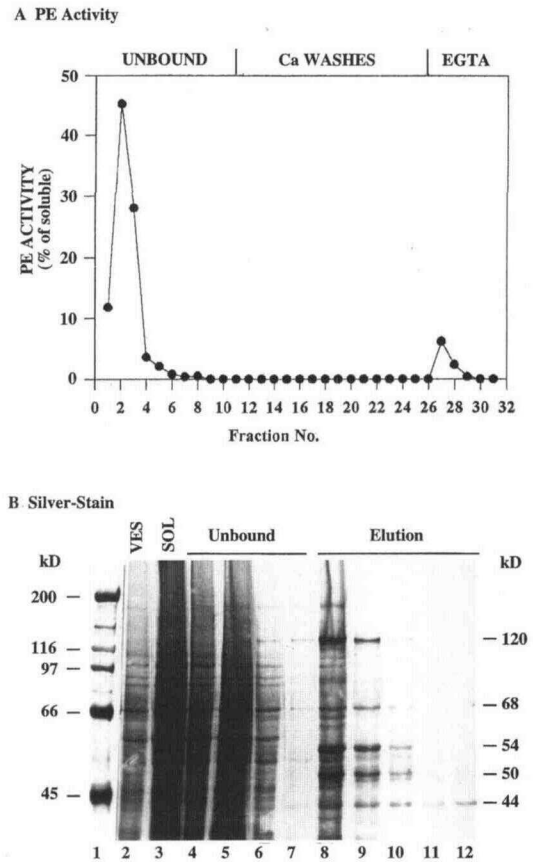

Figure 8. Partial purification of a 120-kD Ca^{2+}-ATPase from carrot suspension culture cells using CaM-Sepharose-affinity chromatography. A, Elution profile of erythrosin-sensitive PE activity. Solubilized protein was allowed to bind to a 2-mL CaM-Sepharose column. To remove unbound material, the column was washed first with buffer containing 5 mM $CaCl_2$ (UNBOUND) and then with decreasing Ca^{2+} from 2 to 0.1 mM (Ca WASHES). Bound protein was eluted with buffer containing 2 mM EGTA (EGTA). Two-milliliter fractions were collected at all times. Data shown are representative of at least seven experiments. B, Proteins purified by CaM-Sepharose-affinity chromatography. Aliquots equal to 1/1000 of the total of EGTA-washed vesicles (VES, lane 2), 1/100 of the total of solubilized protein (SOL, lane 3), 1/10 of fraction 1 (lane 4), and one-half of wash fractions 6, 13, and 26 (lanes 5–7, respectively) and EGTA-eluted fractions 27 to 31 (lanes 8–12, respectively) were separated on a 10% acrylamide gel and visualized with silver staining. Molecular mass standards (lane 1) were loaded to approximately 0.4 μg/band.

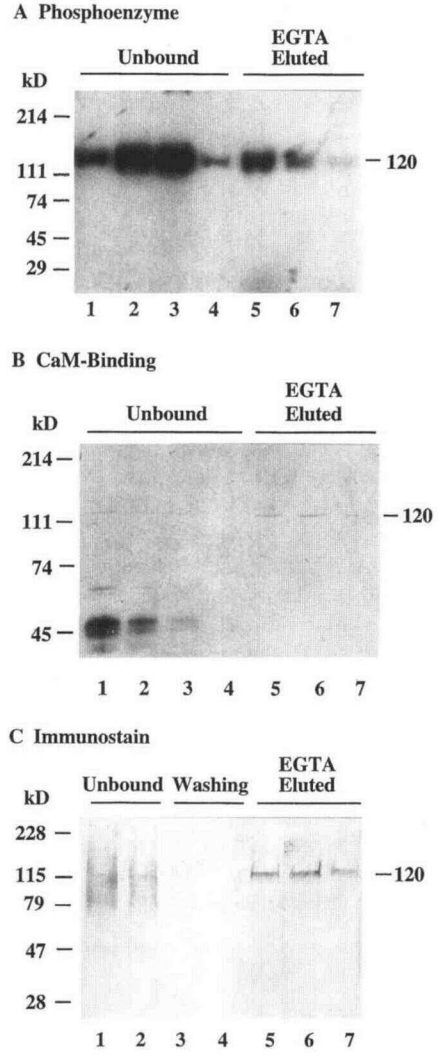

Figure 9. Co-elution of a 120-kD Ca^{2+}-dependent phosphoprotein (A), a 120-kD CaM-binding protein (B), and an immunoreactive 120-kD protein with antibodies to a Ca^{2+}-ATPase (C). Solubilized proteins were allowed to bind to a CaM-affinity column and then eluted as described in Figure 8. Fraction 1 to 4 containing 2 mL each (lanes 1–4) were eluted in the presence of Ca^{2+} and refer to the initial "Unbound" fractions (A and B). Lanes 5 to 7 correspond to fractions 27 to 29 eluted by EGTA (Bound). A, Steady-state ^{32}P-PE levels were determined with 2 nM ATP in the presence of 100 μM La^{3+} at 2 min. PE formed from 5% of fractions 1 to 4 (lanes 1–4, respectively) and 36% of fractions 27 to 29 (lanes 5–7, respectively) are shown. No PE was detected in fractions 6 to 26 when 40% of the fraction was assayed. No additional phosphoproteins were observed in other parts of the gel. B, Binding to biotinylated CaM was determined using 1% of fractions 1 to 4 (lanes 1–4, respectively) and 10% of fractions 27 to 29 (lanes 5–7, respectively). Fractions 6 to 26 had no detectable CaM-binding activity. C, Immunostaining with the antibody to a CaM-stimulated Ca^{2+}-ATPase from cauliflower (1:500). One percent of the un- bound (lanes 1 and 2) and 30% of the washed (lanes 3 and 4) and bound fractions (lanes 5–7) were used for immunostaining. Lanes 3 and 4 refer to fractions eluted with 2 and 0.1 mM Ca^{2+} washes, respectively.

date (Table IV), which is consistent with the activity of a Ca^{2+}-ATPase. The partially purified PE activity was inhibited by erythrosin B (inhibitor concentration required for 50% inhibition was approximately 2 μM) (Table III) and did not require added Mg^{2+}. The steady-state level of PE was strongly enhanced by La^{3+} (Table IV), a property that is considered diagnostic for the animal PM-type Ca^{2+}-ATPase (Carafoli, 1991).

Association of a 120-kD Ca^{2+}-ATPase with Several Membrane Fractions

Immunostaining with an antibody to a cauliflower Ca^{2+}-ATPase showed that a Ca^{2+}-ATPase of 120 kD was distributed broadly in endomembranes (25–32% Suc) from carrots (Fig. 10), similar to the distribution of GTP-dependent PE (Fig. 5) and of CaM-binding activities (Fig. 6B). In the PM fraction another polypeptide of 127 kD was strongly immunoreactive with anti-Ca^{2+}-ATPase (Fig. 10). The ability to bind to CaM (Fig. 6B) suggested that the 127-kD protein could be an additional CaM-stimulated Ca^{2+} pump. Weak immunoreactivity of a 120-kD polypeptide in the vacuolar membrane fraction (15–24% Suc) and the appearance of an immunoreactive 64-kD protein could be due to proteolysis (Fig. 10), since material at the top of the gradient was directly exposed to soluble hydrolytic enzymes. In all other fractions, the antibody reacted most strongly with a major polypeptide of 120 kD in the endomembranes and with a 127-kD protein in the PM.

DISCUSSION

Discrimination between Two Major Types of Ca^{2+} Pumps: PM-Type and ER-Type

Although two major types of Ca^{2+} pumps, the PM-type and the SER-type, are well documented in animals (Schatzmann, 1989; Carafoli, 1991), the distinction between the different Ca^{2+} pumps in plants has been less certain (Evans et al., 1991). The major reasons are (a) the pumps are very similar as P-type ATPases; (b) there is a lack of distinguishing biochemical features; and (c) each pump type is not necessarily restricted to one particular organelle or membrane. Here we have demonstrated the distinction between the PM-type and ER-type Ca^{2+} pumps in one plant material, even when membrane fractions contain a mixture of pump types.

Taking advantage of differential substrate specificities, differential CaM sensitivities, and inhibitor sensitivities, we were able to discriminate between two major classes of Ca^{2+} pumps in carrots (Table V): (a) PM-type Ca^{2+} pumps, which were characterized by the ability to hydrolyze GTP as well as ATP and by their sensitivity to CaM (Fig. 1), and (b) an ER-type Ca^{2+} pump, which preferred ATP as a substrate, and was blocked by cyclopiazonic acid but was insensitive to CaM (Fig. 1C). The separation of these two pump types on the distinct membrane compartments was suggested by the selective stimulation of oxalate on the ER-type, but not the PM-type, Ca^{2+} accumulation (Fig. 3; Table I). This simple model of two pump types is consistent with and extends the results from several laboratories (Briskin, 1990; Hsieh et al., 1991; Thomson et al., 1993; Bush and Wang, 1995). The differential sensitivities to erythrosin B (see refs. cited in Briskin, 1990) and differential affinities for Ca^{2+} (Bush and Wang, 1995) are additional, useful features that were not explored in this study. However, unlike animal PM-Ca^{2+}-ATPases, GTP-dependent PM-type Ca^{2+} pumping (e.g. a 120-kD pump) in plants was localized on several membranes, including the vacuole and PM. To our knowledge, we have demonstrated the first selective inhibition by cyclopiazonic acid of an ER-type Ca^{2+} pump from plants (Fig. 3). Purified ER membranes from previous studies were either not tested for (Bush and Wang, 1995) or failed to show any cyclopiazonic acid sensitivity (Thomson et al., 1993).

Because the two types of pumps were characterized by their biochemical features and not by their membrane location, the terms ER- and PM-type are used here to reflect their key biochemical properties (Table V). These terms are also useful in classifying plant gene products that are homologous to either animal SER- or PM-type Ca^{2+} pumps (Wimmers et al., 1992). Thus, the terms ER-type or PM-type do not necessarily imply an absolute association with either the ER or the PM, respectively. Furthermore, each type may be represented by several

Table III. *Ca^{2+} dependence and erythrosin sensitivity of the PE activity from Ca^{2+}-ATPase partially purified by CaM-affinity chromatography*

PE activities were determined as steady-state PE levels for a 100-μL aliquot of the bound/EGTA-eluate fraction (no. 27 in Fig. 8A) by adding inhibitors/salts as indicated and then 2 nM [^{32}P]ATP (1 μCi) to start the reaction (final volume 125 μL). Since EGTA-eluted fractions contained 2 mM EGTA, 10 mM $CaCl_2$ and 100 μM $LaCl_3$ were added to assay PE activities. Specific activities in fmol/mg were calculated using an estimate of 0.1 μg/100 μL protein. Data are from one experiment that is representative of three.

Condition	[^{32}P]PE Activity			
	Total	Ca^{2+}-dependent		
	cpm	*cpm*	*fmol/mg*	*%*
2 mM EGTA	1,816	0		
2 mM EGTA + 10 mM $CaCl_2$	10,567	8,751	3540	
Erythrosin B (μM)				
0	10,567	8,751	3540	100
0.1	11,869	10,053	3960	115
1	6,967	5,151	2030	59
5	2,504	688	270	8
10	1,610	0	0	0

independent but related pumps. Thus, there are multiple PM-type Ca^{2+} pumps in which one resided on the vacuolar membrane and another on the PM (Figs. 1 and 3), although it was unclear whether the pumps were distinct polypeptides encoded by separate genes or whether they were products of one gene. As a step toward identifying a PM-type Ca^{2+} pump, we have solubilized and partially purified a CaM-binding Ca^{2+} pump.

Partial Purification of a 120-kD CaM-Binding Ca^{2+} Pump from Endomembranes

To purify a Ca^{2+}-ATPase from endomembranes, we monitored the enzyme by its ability to form a phosphorylated intermediate (PE). Using this sensitive assay, we had characterized a Ca^{2+}-ATPase in low-density membranes, prior to any attempt at purification (Chen et al., 1993). We had concluded that a 120-kD Ca^{2+}-ATPase was like the animal PM-type Ca^{2+}-ATPase based on the following: (a) PE formation is stimulated by La^{3+}, (b) PE formation and Ca^{2+} transport are stimulated by CaM, (c) PE formation is not dependent on exogenous Mg^{2+}, and (d) PE formation is insensitive to thapsigargin or cyclopiazonic acid, inhibitors of the SER-type Ca^{2+}-ATPase. The Ca^{2+}-ATPase that was partially purified in the present study has properties similar to that membrane-bound Ca^{2+}-ATPase of 120 kD from carrot: formation of PE is dependent on Ca^{2+}, stimulated by La^{3+}, and sensitive to erythrosin B and does not require added Mg^{2+} (Tables III and IV). However, the pump may have been slightly modified during purification, hence, the decreased sensitivity to erythrosin (inhibitor concentration required for 50% inhibition of 2 versus <0.1 μM) and the decreased effect of CaM on steady-state PE (no effect versus 25% stimulation). The purified fraction contained a 120-kD polypeptide that bound to CaM and reacted with antibodies against a CaM-stimulated Ca^{2+} pump from cauliflower (Fig. 9). These results demonstrate that we have partially purified a PM-type Ca^{2+} pump of 120 kD.

Weak Binding of a CaM-Stimulated Ca^{2+} Pump to CaM and Presence of a CaM-Insensitive Ca^{2+} Pump in Endomembranes

It is interesting that a majority of the Ca^{2+}-ATPase PE activity did not bind to the CaM-affinity column (Fig. 8A),

Table IV. *PE formation of the partially purified Ca^{2+}-ATPase was stimulated by La^{3+} (100 μM) and inhibited by vanadate*

Activity in the EGTA-eluted fractions was assayed as in Table III.

Condition	EB[a]-Sensitive	PE	Activity
	cpm	*fmol/mg*	*%*
No Mg			
− $LaCl_3$	0	0	0
+ $LaCl_3$	8957	3530	100
1 mM Mg			
− $LaCl_3$	0	0	0
+ $LaCl_3$	7322	2890	100
+ $LaCl_3$ + 100 μM vanadate	2636	1040	36

[a] EB, Erythrosin B.

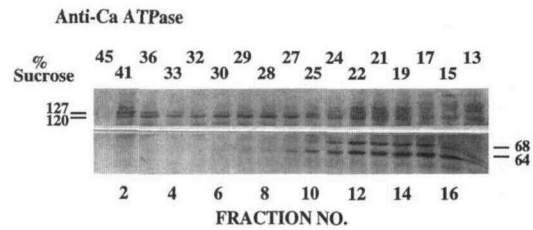

Figure 10. Distribution of a 120-kD Ca^{2+}-ATPase in several endomembranes from carrot cells. The post-mitochondrial supernatant was separated with a linear Suc gradient as in Figure 6, and each fraction was analyzed by SDS-PAGE. Blots were immunostained with antibodies to a CaM-stimulated Ca^{2+}-ATPase from cauliflower (1:500).

in spite of our efforts to increase the binding by modifying conditions. The presence of a large amount of activity remaining unbound may be due to one or more of the following possibilities: (a) insufficient CaM-binding sites on the column; (b) weak binding due to low affinity of the Ca^{2+}-ATPase for CaM, endogenous CaM remaining bound to the Ca^{2+}-ATPase, and proteolysis of the CaM-binding domain; and (c) the presence of other Ca^{2+}-ATPases that do not bind to CaM and that account for part of the PE activity. Possibility (a) seemed unlikely, since the unbound fraction did not bind to a second CaM-affinity column (not shown). Weak binding between CaM and a carrot Ca^{2+} pump (possibility [b]) is suggested by the concentration of CaM required for one-half maximal stimulation. The $K_{0.5}$ of Ca^{2+} transport in endomembrane vesicles is about 200 nM CaM (Hsieh et al., 1991; Liss and Weiler, 1994), which is 40- to 200-fold higher than the $K_{0.5}$ of 1 to 5 nM of the erythrocyte PM-type Ca^{2+}-ATPase (Schatzmann, 1982; Carafoli, 1992). Furthermore, a 120-kD polypeptide that bound to CaM and reacted with an antibody to a cauliflower CaM-stimulated Ca^{2+}-ATPase was detected in fractions that did not bind to the CaM column (Fig. 9, B and C). Thus, weak binding of a Ca^{2+} pump to CaM could account in part for the unbound PE (Table II; Fig. 9A).

Compelling evidence supports the idea for another type of Ca^{2+} pump in plant endomembranes, especially the ER. First, an ER-type Ca^{2+} pump activity was CaM-insensitive, preferred ATP as a substrate (Figs. 1C and 3A; Buckhout, 1984; Thomson et al., 1993; Bush and Wang, 1995), and was inhibited by cyclopiazonic acid (Fig. 4), a specific inhibitor of animal SERCa pumps (Siedler et al., 1989). Second, the extent of La^{3+} stimulation of PE formation was much higher in the purified fraction (absolute requirement; Table IV) than in the membrane-bound enzyme (2- to 3-fold) (Chen et al., 1993). If we assume that plant Ca^{2+} pumps are similar to animal Ca^{2+} pumps, PE formation of the PM-type, but not ER-type, pump is stimulated by La^{3+}. Thus, an increase in La^{3+} stimulation could indicate an enrichment of the PM-type pump relative to the ER-type pump. Third, like tobacco (Perez-Prat et al., 1992) and tomato (Wimmers et al., 1992), carrot possessed a homolog of animal SERCa pumps. Preliminary studies indicated that the C terminus of the carrot SERCa homolog did not bind to CaM (F. Liang, unpublished data). All of these results support the notion that part of the unbound PE activity

originated from plant ER-type Ca^{2+} pump(s). Thus, monitoring Ca^{2+} pump(s) by PE formation is extremely sensitive, although it could reflect activity from more than one type of Ca^{2+} pump.

Surprisingly, the tomato Lycopersicon Ca^{2+}-ATPase protein was recently located on the tonoplast and the PM rather than the ER (Ferrol and Bennett, 1996). It is possible that the antibody was not ER-type specific and was also recognized by a domain from PM-type Ca^{2+} pumps. Alternatively, unlike carrot, ER-type Ca^{2+} pumps could also reside on the PM and the vacuole of tomato.

Role of a CaM-Stimulated 120-kD Ca^{2+} Pump in Endomembranes

So far only a few CaM-stimulated Ca^{2+}-ATPases (of 120 and 111–115 kD) have been purified from low-density endomembranes of plants (this work; Askerlund and Evans, 1992; Askerlund, 1996); however, numerous reports of CaM-stimulated Ca^{2+} transport in endomembranes (Brauer et al., 1990; Hsieh at al., 1991; Gilroy and Jones, 1993; Bush and Wang, 1995) illustrate that this is a common feature in plants. Recent studies in corn roots have attributed a low-density, CaM-stimulated Ca^{2+} pump to the vacuole alone (Gavin et al., 1993; Pfeiffer and Hager, 1993); however, in the tendrils of *Bryonia*, CaM-stimulated Ca^{2+} transport was found in purified ER after ribosomes had been stripped (Liss and Weiler, 1994). Thus, a PM-type Ca^{2+} pump can be associated with the ER of certain plant cells, although it is unclear whether this pump is resident on the ER or whether it is synthesized there and then destined for other membranes of the secretory system, or both. The possibility that a CaM-stimulated Ca^{2+} pump participates in secretion is suggested by the parallel distribution of a 120-kD Ca^{2+} pump and a secreted glycoprotein EP1 (van Engelen et al., 1991) in endomembranes (I. Hwang and H. Sze, unpublished data). Notably, endomembrane CaM-stimulated Ca^{2+} pumping is often found in actively proliferating or highly secretory cells or both, where the regulation of intraluminal $[Ca^{2+}]$ and cytosolic $[Ca^{2+}]$ are critical for proper protein folding, secretion, and membrane fusion (Battey and Blackbourn, 1993).

Are CaM-Stimulated Ca^{2+} Pump(s) on the PM Distinct from a 111- to 120-kD Pump Purified from Endomembranes?

Polypeptides of 125–127 kD visible in the PM fraction of carrot by CaM binding or by immunostaining with anti-Ca^{2+}-ATPase (Figs. 6B and 10) are likely candidates for CaM-stimulated Ca^{2+} pump(s) in addition to a 120-kD Ca^{2+} pump on endomembranes (Figs. 6B and 10). The molecular mass of PM-bound Ca^{2+} pumps is usually larger than the 111- to 120-kD pump purified from endomembranes (this study; Askerlund, 1996). In radish seedlings a CaM-binding Ca^{2+} pump localized only to the PM has a molecular mass of 124 to 133 kD, based on a PE formed with [^{32}P]GTP and by labeling with fluorescein isothiocyanate (Rasi-Caldogno et al., 1995). In red beets a 124-kD PE was associated with the PM, whereas a 119-kD PE was bound to ER (Thomson et al., 1993). In cauliflower a 116-kD polypeptide in a heavy-density fraction could be a PM-bound Ca^{2+} pump, judging by its reactivity with antibodies to the CaM-stimulated 111-kD Ca^{2+} pump from endomembranes (Askerlund, 1996). These results from several laboratories suggest that a CaM-stimulated Ca^{2+} pump from the PM is distinct from a pump on low-density endomembranes. The presence of PM-type Ca^{2+} pumps that do not bind directly to CaM (Cunningham and Fink, 1994; Liss and Weiler, 1994) is also considered. Multiple genes appear to encode the PM-type Ca^{2+} pump homologs from Arabidopsis (EST Database and J. Harper, personal communication); thus functional expression of individual genes is a critical step toward understanding their transport and regulatory properties.

Summary

The ability to distinguish between two types of Ca^{2+} pumps biochemically clarifies, in part, the apparent incon-

Table V. *Distinction between ER-type and PM-type Ca^{2+} pumps from carrots and molecular masses of Ca^{2+} pumps from various plants*

Properties are mainly derived from this study and supported by earlier studies.

Plant and Molecular Mass	PM-Type	ER-Type
Carrot[a,b,c]		
Substrate preferred	ATP ≥ GTP	ATP
CaM (2 μM)	2- to 4-fold stimulation[b]	Not stimulated
Oxalate on Ca^{2+} accumulation into native vesicles	No effect	4- to 10-fold stimulation[b]
Cyclopiazonic acid	No effect	Inhibition (100 nmol/mg)
Membrane location (density gradient)	Endomembrane: e.g. ER, vacuolar, secretory vesicles; PM	ER mostly Golgi
Molecular mass of Ca^{2+} pumps from various plants		
Carrot (this study)	Endomembrane: ~120 kD (PE); PM: 127 KD	ER: ~120 kD (PE)
Cauliflower[d]	Endomembrane: 111 kD; PM: 116 kD	
Radish[e]	PM: 124–133 kD	
Red beet[f]	PM: 124 kD (PE)	ER: 119 kD (PE)

[a] Bush and Sze, 1986. [b] Hsieh et al., 1991. [c] Chen et al., 1993. [d] Askerlund, 1996. [e] Rasi-Caldogno et al., 1995. [f] Thomson et al., 1993.

sistencies and confusion about plant Ca^{2+}-ATPases (see introduction). We suggest that all plant cells possess both types of pumps in varying proportions. The membrane distribution and the relative activities of each Ca^{2+} pump change, depending on the stage of development and the functions of the cell, tissue, and organ. In carrot suspension cells a PM-type Ca^{2+} pump on the PM is especially prominent in cells at the stationary growth phase (not shown); whereas a PM-type pump on endomembranes is active in cells during exponential growth. The contribution of either ER- or PM-type pump activity can be determined in membranes containing a mixture of pumps using a set of criteria based on substrate preference, inhibitor sensitivity, and stimulation by CaM or oxalate. Importantly, these properties are essential for relating Ca^{2+} pump activities of plant genes that are expressed in yeast with native pump functions.

ACKNOWLEDGMENTS

We thank Dr. Imara Perera and Feng Liang for stimulating discussions and Frank H. Chen for initiating the project. We acknowledge Drs. Paul Bottino and Todd Cooke (University of Maryland, College Park) for the use of their tissue culture facilities. We especially want to thank V. Ling and S. Assmann for their help with ^{125}I-CaM-binding assays during the preliminary phase of this study (1993). We gratefully acknowledge R.T. Leonard, M. Chrispeels, M. Maeshima, and P. Askerlund for providing antibodies to the PM H^+-ATPase, BiP, VM 23, and Ca^{2+}-ATPase, respectively.

Received July 11, 1996; accepted November 7, 1996.
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LITERATURE CITED

Askerlund P (1996) Modulation of an intracellular calmodulin-stimulated Ca^{2+}-pumping ATPase in cauliflower by trypsin. The use of Calcium Green-5N to measure Ca^{2+} transport in membrane vesicles. Plant Physiol **110:** 913–922

Askerlund P, Evans DE (1992) Reconstitution and characterization of a calmodulin-stimulated Ca^{2+}-pumping ATPase purified from *Brassica oleracea* L. Plant Physiol **100:** 1670–1681

Battey NH, Blackbourn HD (1993) The control of exocytosis in plant cells. New Phytol **125:** 307–331

Bergeron JM, Brenner MB, Thomas DY, Williams DB (1994) Calnexin: a membrane bound chaperone of the endoplasmic reticulum. Trends Biochem Sci **19:** 124–128

Brauer D, Schubert C, Tsu SI (1990) Characterization of a Ca^{2+}-translocating ATPase from corn root microsomes. Physiol Plant **78:** 335–344

Briskin DP (1990) Ca^{2+}-translocating ATPase of the plasma membrane. Plant Physiol **94:** 397–400

Buckhout TJ (1984) Characterization of Ca^{2+} transport in purified endoplasmic reticulum membrane vesicles from *Lepidium sativum* L. roots. Plant Physiol **76:** 962–967

Bush DR, Sze H (1986) Calcium transport in tonoplast and endoplasmic reticulum vesicles isolated from cultured carrot cells. Plant Physiol **80:** 549–555

Bush DS (1995) Calcium regulation in plants cells and its role in signaling. Annu Rev Plant Physiol Plant Mol Biol **46:** 95–122

Bush DS, Wang T (1995) Diversity of calcium efflux transporters in wheat aleurone cells. Planta **197:** 19–30

Camacho P, Leihleiter JD (1993) Increased frequency of calcium waves in *Xenopus laevis* oocytes that express a Ca^{2+}-ATPase. Science **260:** 226–229

Carafoli E (1991) Calcium pump of the plasma membrane. Physiol Rev **71:** 129–153

Carafoli E (1992) The Ca^{2+} pump of the plasma membrane. J Biol Chem **267:** 2115–2118

Chen FH, Ratterman DM, Sze H (1993) A plasma membrane-type Ca^{2+}-ATPase of 120 kilodaltons on the endoplasmic reticulum from carrot (*Daucus carota*) cells. Properties of the phosphorylated intermediate. Plant Physiol **102:** 651–661

Cunningham KW, Fink GR (1994) Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking *PMC1*, a homology of plasma membrane Ca^{2+}-ATPases. J Cell Biol **124:** 351–363

Dupont FM, Bush DS, Winddle JJ, Jones RL (1990) Calcium and proton transport in membrane vesicles from barley roots. Plant Physiol **94:** 179–188

Evans DE, Briars SA, Williams LE (1991) Active calcium transport by plant cell membranes. J Exp Bot **42:** 285–303

Ferrol N, Bennett AB (1996) A single gene may encode differentially localized Ca^{2+}-ATPases in tomato. Plant Cell **8:** 1159–1169

Gavin O, Pilet P-E, Chanson A (1993) Tonoplast localization of a calmodulin-stimulated Ca^{2+} pump from maize roots. Plant Sci **92:** 143–150

Gilroy S, Jones RL (1993) Calmodulin stimulation of unidirectional Ca^{2+} uptake by endoplasmic reticulum of barley aleurone layer. Planta **190:** 289–296

Hsieh WL, Pierce WS, Sze H (1991) Calcium-pumping ATPases in vesicles from carrot cells. Stimulation by calmodulin or phosphatidylserine and formation of a 120 kilodalton phosphoenzyme. Plant Physiol **97:** 1535–1544

Kincaid RL, Billingsley ML, Vaughan M (1988) Preparation of fluorescent, cross-linking, biotinylated calmodulin derivatives and their use in studies of calmodulin-activated phosphodiesterase and protein phosphatase. Methods Enzymol **159:** 605–626

Ling V, Assmann SM (1992) Cellular distribution of calmodulin and calmodulin-binding proteins in *Vicia faba* L. Plant Physiol **100:** 970–978

Liss H, Weiler EW (1994) Ion-translocating ATPases in tendrils of *Bryonia dioica* Jacq. Planta **194:** 169–180

Maeshima M (1992) Characterization of the major integral protein of vacuolar membrane. Plant Physiol **98:** 1248–1254

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant **15:** 473–497

Niggli V, Penniston JT, Carafoli E (1979) Purification of the (Ca^{2+}-Mg^{2+})-ATPase from human erythrocyte membranes using a calmodulin affinity column. J Biol Chem **254:** 955–958

Perez-Prat E, Narasimhan ML, Binzel ML, Botella MA, Chen Z, Valpuesta V, Bressan RA, Hasegawa PM (1992) Induction of a putative Ca^{2+}-ATPase mRNA in NaCl-adapted cells. Plant Physiol **100:** 1471–1478

Pfeiffer W, Hager A (1993) A Ca^{2+}-ATPase and a Mg^{2+}/H^+ antiporter are present on tonoplast membranes from roots of *Zea mays* L. Planta **191:** 377–385

Rasi-Caldogno F, Carnelli A, De Michelis MI (1992) Plasma membrane Ca^{2+}-ATPase of radish seedlings. II. Regulation by calmodulin. Plant Physiol **98:** 1202–1206

Rasi-Caldogno F, Carnelli A, De Michelis MI (1995) Identification of the plasma membrane Ca^{2+}-ATPase and of its autoinhibitory domain. Plant Physiol **108:** 105–113

Rudolph HK, Antebi A, Fink GR, Buckley CM, Dorman TE, LeVitre J, Davidow LS, Mao LS, Moir DT (1989) The yeast secretory pathway is perturbed by mutations in PMR1, a member of a Ca^{2+}-ATPase family. Cell **58:** 133–145

Sambrook JF (1990) The involvement of calcium in transport of secretory proteins from the endoplasmic reticulum. Cell **61:** 197–199

Schatzmann HJ (1982) The plasma membrane calcium pump of erythrocytes and other animal cells. *In* E Carafoli, ed, Membrane Transport of Calcium. Academic Press, London, pp 41–108

Schatzmann HJ (1989) The calcium pump of the surface membrane and of the sarcoplasmic reticulum. Annu Rev Physiol **51:** 473–485

Siedler NW, Jona I, Vegh M, Martinosi A (1989) Cyclopiazonic acid is a specific inhibitor of the Ca^{2+} -ATPase of sarcoplasmic reticulum. J Biol Chem **426:** 17816–17823

Thomson LJ, Xing T, Hall JL, Williams LE (1993) Investigation of the calcium-transporting ATPases at the endoplasmic reticulum and plasma membrane of red beet (*Beta vulgaris*). Plant Physiol **102:** 553–564

van Engelen FA, Sterk P, Booij H, Cordewener JHG, Rook W, van Kammen A, de Vries SC (1991) Heterogeneity and cell type-specific localization of a cell wall glycoprotein from carrot suspension cells. Plant Physiol **96:** 705–712

Weber K, Osborn M (1969) The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. J Biol Chem **244:** 4406–4412

Wimmers LE, Ewing NN, Bennett AB (1992) Higher plant Ca^{2+}-ATPase: primary structure and regulation of mRNA abundance by salt. Proc Natl Acad Sci USA **89:** 9205–9209